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Feeder-free growth of undifferentiated human embryonic stem cells

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Previous studies have shown that maintenance of undifferentiated human embryonic stem (hES) cells requires culture on mouse embryonic fibroblast (MEF) feeders. Here we demonstrate a successful feeder-free hES culture system in which undifferentiated cells can be maintained for at least 130 population doublings. In this system, hES cells are cultured on Matrigel or laminin in medium conditioned by MEF. The hES cells maintained on feeders or off feeders express integrin α6 and β1, which may form a laminin-specific receptor. The hES cell populations in feeder-free conditions maintained a normal karyotype, stable proliferation rate, and high telomerase activity. Similar to cells cultured on feeders, hES cells maintained under feeder-free conditions expressed OCT-4, hTERT, alkaline phosphatase, and surface markers including SSEA-4, Tra 1-60, and Tra 1-81. In addition, hES cells maintained without direct feeder contact formed teratomas in SCID/beige mice and differentiated *in vitro* into cells from all three germ layers. Thus, the cells retain fundamental characteristics of hES cells in this culture system and are suitable for scaleup production.

Human ES cell lines have been successfully derived from the inner cell mass of preimplantation embryos[1,2] and can be maintained *in vitro* for over one year (~250 population doublings), while remaining karyotypically and phenotypically stable[3]. Similar to mouse ES cells, hES cells have the capacity to differentiate into cell types from all three germ layers both *in vitro* and *in vivo*[1–3]. These characteristics indicate that hES cells have the potential to provide an unlimited supply of different cell types for tissue replacement, drug screening, and functional genomics applications. All potential applications, however, depend largely on routine availability of moderate to large numbers of cells, requiring methods amenable to scaleup.

Previous reports indicate that undifferentiated hES cultures require MEF feeder cells to maintain the undifferentiated state[1,2]. In mouse ES cultures, the feeder layer can be replaced by addition of the cytokine, leukemia inhibitory factor (LIF), to the growth medium[4,5]. But LIF does not have this effect in human ES cultures[1,2], and both the derivation and maintenance of human ES cells apparently require the use of feeder cells. Unfortunately, this latter practice is technically challenging for large-scale production of hES cells. To alleviate this difficulty, we have developed a feeder-free culture system for the maintenance and proliferation of hES cells.

Considering that most normal cells require adhesion to an extracellular matrix for survival and growth, and that soluble factors produced by MEF may be important for hES cells, we evaluated the ability of different matrices in MEF-conditioned medium (MEF-CM) to support hES growth. Undifferentiated hES colonies were harvested from hES cultures on feeders and then seeded onto Matrigel or gelatin-coated plates in MEF-CM. The day after seeding, cells on Matrigel attached and formed small colonies that were less compact than hES colonies on feeder layers. Over the next few days, these colonies increased in size and cell number, and became more compact. Cells between the colonies appeared differentiated, so the resulting culture contained very dense undifferentiated colonies surrounded by differentiated cells (Fig. 1B, K). About one week after seeding, the cultures became confluent and ~80% of the surface area had cells with undifferentiated hES morphology. All cell lines tested (H1, H7, H9, and H14) showed similar results. The feeder-free cells had a doubling time of 31–33 h, similar to that reported for hES cells grown on feeders[3]. They also had a normal karyotype as determined by GTG-banding analysis. In addition, the hES cells were successfully cryopreserved in ES medium (see Experimental Protocol) supplemented with 10% dimethyl sulfoxide and 30% KNOCKOUT Serum Replacement using a controlled-rate freezer according to the criteria of recovery of growth and differentiation potential. In contrast, cells seeded onto gelatin in MEF-CM showed poor survival, and the cells that survived appeared differentiated within the first passage. These data indicate that hES cells can be maintained on Matrigel in MEF-CM.

Matrigel contains mostly laminin, collagen IV, and heparan sulfate proteoglycan[6,7]. We also examined individual basement membrane components, including laminin, collagen IV, and fibronectin. Similar to cultures on Matrigel or feeders, cultures maintained in MEF-CM on laminin, fibronectin, or collagen IV contained compact colonies of undifferentiated hES cells, although the cultures on fibronectin or collagen IV generally did not contain as many undifferentiated colonies as the cultures on Matrigel or laminin (Fig. 1A–E). However, cultures on Matrigel or laminin in nonconditioned ES medium had a completely differentiated morphology after two passages (Fig. 1J). Thus, the combination of appropriate matrix proteins, such as Matrigel or laminin, and MEF-CM supports the growth of undifferentiated hES cells.

We also evaluated cell growth on Matrigel or laminin in CM from sources other than MEF cells. Conditioned media from STO (an immortal mouse embryonic fibroblast cell line), NHG190 (a mouse embryonic cell line transfected with hTERT (human telomerase catalytic subunit); unpublished data), BJ5ta (a human foreskin fibroblast cell line immortalized with telomerase)[8], and hTERT-RPE (a human retinal epithelial cell line immortalized with telomerase)[8] were prepared similarly to that from MEF. The effects of the different conditioned media were compared using parallel H9 cultures maintained on Matrigel or laminin. Cells grown in hTERT-RPE-CM differentiated within the first week of culture. Very few colonies with appropriate ES morphology were found in cultures maintained in CM from STO or BJ5ta after 56 days (passage 32 + 7) (Fig. 1H, I). Although some undifferentiated colonies formed in NHG190-CM, they were less confluent compared with those in MEF-CM (Fig. 1F, G). Therefore, only CM from particular cells or cell lines supported hES cell growth. It is likely that these cells are producing factors that support hES growth, but we cannot rule out the possibility that they are removing toxic factors from the medium.

The above results indicate that laminin and MEF-CM are able to maintain hES cells in the undifferentiated state. Laminin is the first extracellular matrix protein expressed in two- to four-cell stage mouse embryos and is a major component of the extracellular matrix of all basal laminae in vertebrates[9,10]. Through interaction with integrin heterodimers such as α1β1, α2β1, α3β1, α6β1, and α6β4 on the cell surface, laminin induces signals for promoting cell adhesion, growth, and migration. Among these integrins, only α6β1 and α6β4 are specific for laminin, whereas the others also interact with other matrix proteins, such as collagen[11]. Laminin receptor was found to be highly expressed on murine ES and embryonal carcinoma cells[12,13]. To determine whether laminin receptors are expressed on hES cells and

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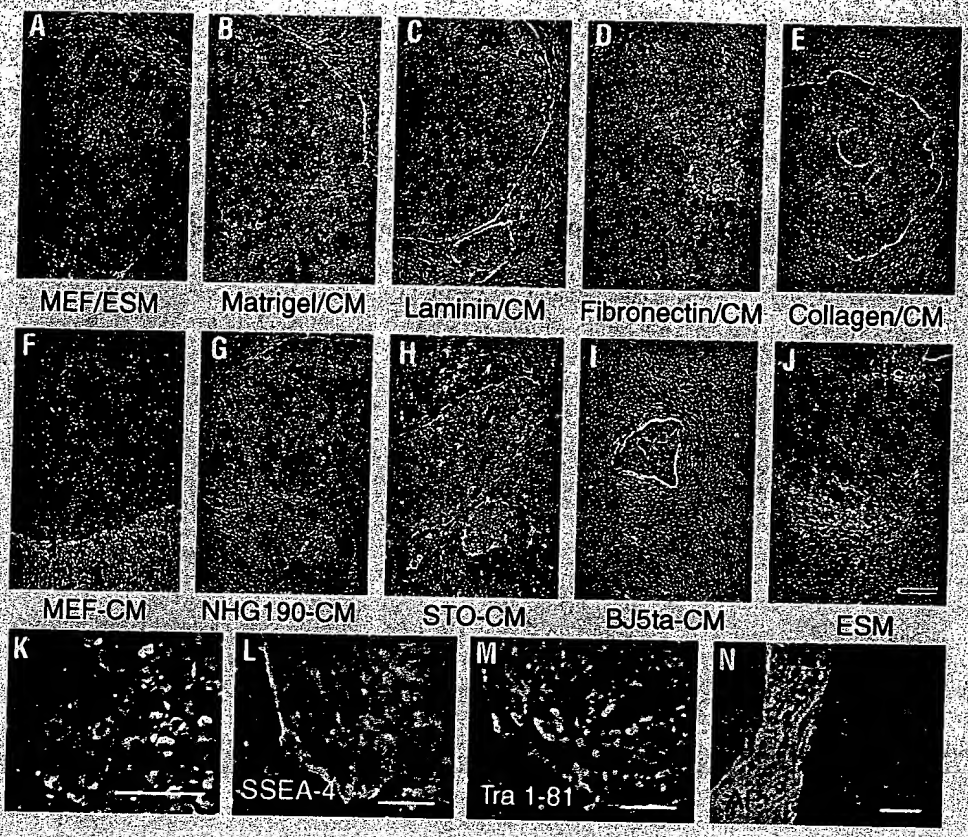


Figure 1. (A–K) Morphology of feeder-free hES cells. H1 cells (passage 46 + 6, 40 days) cultured on feeders in ES medium (MEF/ESM) (A), or on Matrigel (B), laminin (C), fibronectin (D), or collagen IV (E) in MEF-CM. H9 cells (passage 32 + 7) maintained for 56 days on Matrigel in medium conditioned by MEF (F), NHG190 (G), STO (H), or BJ5ta (I), as well as nonconditioned ES medium (ESM) (J). (A–J) Bar, 300 μm. (K) High magnification of hES cells on Matrigel in MEF-CM. Bar, 50 μm. (L–N) Detection of surface marker expression. SSEA-4 (L) and Tra 1-81 (M) detected by immunostaining, and alkaline phosphatase (AP) (N) detected with Vector substrate (Vector Laboratories, Inc., Burlingame, CA) in H1 cells (passage 39 + 4) maintained on Matrigel in MEF-CM for 25 days. (L–N) Bar, 100 μm.

whether culturing hES cells on laminin or Matrigel changes their expression, we examined the expression of integrins by flow cytometry using H1 cells maintained on feeders, Matrigel, or laminin. We found that hES cells expressed high levels of α6 and β1, moderate levels of α2, and low levels of α1, α2, and α3, and β4 (Fig. 2A). Nearly all cells (97–99%) were positive for both α6 and β1 (Fig. 2A), which may form a dimer specific for laminin. There was no apparent difference in the integrin expression profile among the culture conditions, indicating that maintaining the hES cells in the absence of feeders did not alter integrin expression. The observed predominant expression of integrin α6 and β1 in these cells is consistent with the finding that laminin is superior to collagen or fibronectin in long-term maintenance of undifferentiated hES cells. Together, these results suggest that the laminin-specific receptor may be important for the interaction of hES cells with the matrices.

To determine whether the feeder-free hES cells retain other features of cells grown on feeders, we first evaluated the cells for the expression of some known, though not yet definitive, surface markers for undifferentiated hES cells[1]. As seen for the cells on feeders, stage-specific embryonic antigen 4 (SSEA-4) and tumor rejection antigens Tra 1-81 (Fig. 1L, M) and Tra 1-60 (data not shown) were detected in the hES colonies in MEF-CM on Matrigel, but not in the differentiated cells in between the colonies. In addition, there was very little expression of SSEA-1, a glycolipid normally not expressed by undifferentiated hES cells maintained on feeders[1]. Cells on Matrigel also showed strong alkaline phosphatase enzyme activity (Fig. 1N). Similar results were found for cells on laminin in CM. These data indicate that the feeder-free cultured cells express surface markers characteristic of undifferentiated hES cells.

The POU transcription factor, octamer-binding transcription factor 4 (OCT-4)[2] and the catalytic component of telomerase, hTERT (unpublished observation), are expressed in undifferentiated hES cells and downregulated upon differentiation. Using a semiquantitative RT-PCR analysis, we found that H1 cells maintained on Matrigel in MEF-CM or on laminin in MEF-CM expressed OCT-4 (Fig. 2B), whereas cells maintained on Matrigel in unconditioned ES medium did not express OCT-4 or hTERT (data not shown). In addition, H1 cells on fibronectin or collagen IV expressed low levels of OCT-4 (Fig. 2B). Examination of hTERT expression by RT-PCR showed a pattern very similar to that of OCT-4 (Fig. 2B). We also found that all cultures maintained on feeders or off feeders showed positive telomerase activity (Fig. 2C). Therefore, the hES cells maintained in the feeder-free system have relatively high levels of OCT-4 and telomerase activity, similar to undifferentiated hES maintained on feeders[1–3].

We also evaluated the capacity of the cultured hES cells to differentiate. *In vitro* differentiation was induced in H1 cells maintained in CM on Matrigel, laminin, fibronectin, or collagen IV. Cells maintained in any of these conditions readily formed embryoid bodies, with a heterogeneous morphology including beating cells in the outgrowths. Immunocytochemical analysis of these cultures identified neurons and cardiomyocytes by the expression of β-tubulin III and cardiac troponin I (cTnI), respectively. In addition, positive staining for α-fetoprotein (AFP) was obtained, indicating the presence of endoderm cells (see Fig. 3 for cells maintained on Matrigel; data are not shown for cells cultured under other conditions). Similar results were observed in feeder-free cultures of H7 and H9 cells. Thus, cells maintained in the feeder-free condition can differentiate into various cell types *in vitro*.

To examine *in vivo* differentiation of the hES cells, we tested four cell lines, H1, H7, H9, and H14, grown on Matrigel in MEF-CM for their ability to form teratomas in severe combined immunodeficiency (SCID)/beige mice. Like cells maintained on feeders, cells cultured without feeders generated complex teratomas with various differentiated cells (Fig. 3). Similar types of cells were observed in teratomas derived from cells maintained on feeders and on laminin (data not shown). Therefore, the feeder-free hES cells were able to differentiate into cell types of all germ layers both *in vitro* and *in vivo*.

At the time of this writing, four hES cell lines have been successfully cultured on Matrigel in MEF-CM. The longest culture has been maintained for over ~130 population doublings (180 days). All long-term cultured cells retained the characteristic morphology and karyotype of hES cells, and expressed markers such as SSEA-4, Tra 1-81, OCT-4, and hTERT. Furthermore, they consistently differentiated into many types of cells including neurons, hepatocytes, and cardiomyocytes during culture for ~130 population doublings. Therefore, the feeder-free hES cells appear equiva-

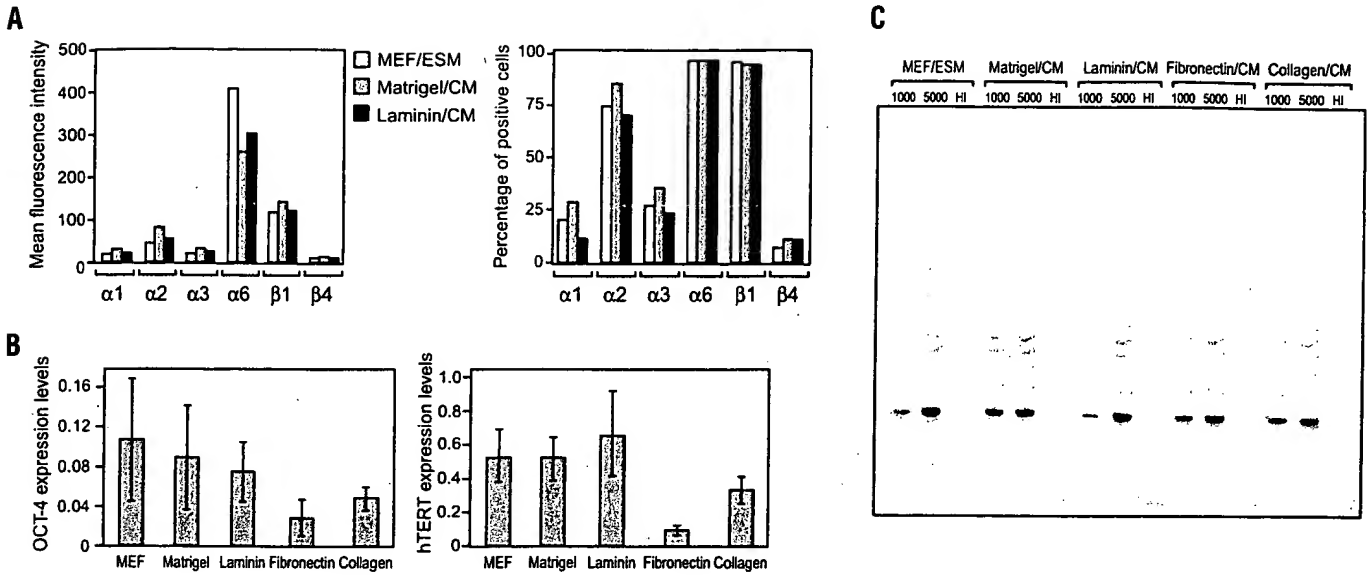
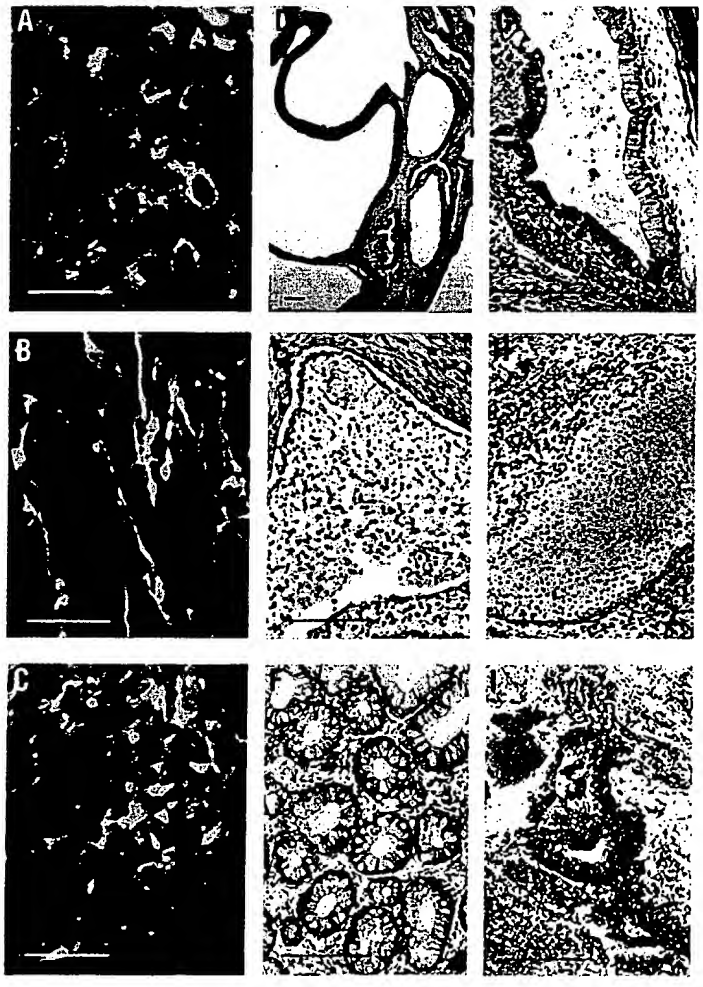


Figure 2. (A) Integrin expression in H1 cells (passage 46 + 7) maintained on feeders in ESM (MEF/ESM) or on Matrigel or laminin in MEF-CM for 42 days. Similar results were obtained in two separate experiments. (B) RT-PCR quantitation of the expression of OCT-4 and hTERT in H1 cells maintained on different matrices in MEF-CM for 21 days (passage 46 + 4). (C) Telomerase activity of H1 cells (passage 46 + 6) maintained on feeders (MEF/ESM) or different matrices in MEF-CM for 40 days. Three lanes were run for each condition. The left lane represents ~1,000 cells, the middle lane represents 5,000 cells, and the right lane is the heat-inactivated control.



lent to hES cells maintained on feeders. The system described here should be useful for generating the large number of hES cells necessary for therapeutic and other applications.

Experimental protocol

Human ES culture. Initial hES cell lines were maintained on feeders in ES medium, which contains 80% KNOCKOUT–Dulbecco's modified Eagle's medium (KO-DMEM), 20% KNOCKOUT serum replacement (Invitrogen, Carlsbad, CA), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 4 ng/ml human basic fibroblast growth factor (hbFGF). Cultures were passaged as cells became confluent (i.e., about once a week) by incubation in 200 units/ml collagenase IV for ~5–10 min at 37°C, dissociated, and then seeded at ~90,000–170,000 cells/cm² onto matrix-coated plates in CM prepared from MEF as follows. MEF cells were harvested and irradiated with 40 Gy, and seeded at ~55,000 cells/cm² in MEF medium. After at least 4 h, the medium was exchanged with ES medium (0.5 ml/cm²). CM was collected daily and supplemented with an additional 4 ng/ml of hbFGF before feeding hES cells. MEF cells were again fed with ES medium daily and used for 7–10 days for CM collection. The CM could also be frozen for storage at –20°C for one month and thawed for later use with no significant difference from fresh CM in supporting cell growth (tested in three hES cell lines). Passage numbers are represented as $x + y$, where x is the passage at which the cells were removed from feeders and y is the number of passages in feeder-free conditions.

Plate coating. Plates were incubated with Matrigel (Becton Dickinson, Bedford, MA) diluted 1:20 in cold KO-DMEM, or laminin (20 μg/ml), collagen IV (10 μg/ml), or fibronectin (5 μg/ml) (all from Sigma, St. Louis, MO) diluted in PBS at 4°C for at least overnight or at room temperature for 1 h.

Figure 3. Differentiation of feeder-free hES cells. (A–C) *In vitro* differentiation of H1 cells (passage 46 + 4) maintained on Matrigel in MEF-CM for 26 days. Positive immunoreactivity was identified for AFP (A), β-tubulin III (B), and cTnI (C), indicating the presence of endoderm, ectoderm, and mesoderm, respectively. Bar, 50 μm. (D–I) Teratomas derived from H7 cells (passage 19 + 11) maintained on Matrigel in MEF-CM for 66 days. Cystic epithelial structures (D), dental component (E), cartilage (H), and glandular (F), epithelial (G), or neural (I) components identified in the teratomas. Bar, 200 μm.

Immunostaining. SSEA-4 and Tra 1-81 immunostaining was carried out similarly as described and detected with fluorescein isothiocyanate (FITC)-labeled secondary antibodies. For β-tubulin III or AFP immunostaining, cells were fixed in 4% paraformaldehyde at room temperature for 20 min followed by permeabilization for 2 min in 100% ethanol. For cTnI staining, cells were fixed in methanol/acetone (3:1) at –20°C for 20 min. Monoclonal antibodies against β-tubulin III or AFP (Sigma), or cTnI (Spectral Diagnostic INC, Toronto, ON, Canada) were used.

Flow cytometry. Cells were stained with a panel of integrin-specific antibodies by the laminin-specific integrins investigator kit (Chemicon International, Inc., Temecula, CA) and analyzed on FACScalibur Flow Cytometer (Becton Dickinson, San Jose, CA) using CellQuest software.

RT-PCR analysis of OCT-4 and hTERT. Expression of OCT-4 and hTERT was measured by semiquantitative RT-PCR. RNA was prepared with the QIAGEN RNeasy kit (QIAGEN, Valencia, CA). Before reverse transcription, RNA samples were digested with DNase I to remove contaminating genomic DNA. Standard reverse-transcription reactions were performed with 500 ng total RNA using random hexamers as primers and Superscript II reverse transcriptase (GIBCO BRL, Rockville, MD). Quantification of individual gene products using QuantumRNA Alternate18S Internal Standard primers (Ambion, Austin, TX) as a control was performed according to the manufacturer's instructions. Briefly, the linear range of amplification of a particular primer pair was determined, then coamplified with the appropriate mixture of alternate 18S primers:competimers to yield PCR products with coinciding linear ranges. OCT-4 primers (sense, 5′-CTTGCTGGAGAAGTGGGTG-GAGGAA-3′; antisense, 5′-CTGCAGTGTGGGTTTCGGGCA-3′) were amplified in the presence of a 1:4 ratio of 18S primers:competimers for 19 cycles. HTERT primers (sense, 5′-CGGAAGAGTGTCTGGAGCAA-3′; antisense, 5′-GGATGAAGCGGAGTCTGGA-3′) were amplified in the presence of a 1:12 ratio of 18S primers:competimers for 34 cycles. Cycles consisted of 30 min denaturation at 94°C, 30 min annealing at 60°C, and 30 min extension at 72°C. Before addition of AmpliTaq (Roche, Indianapolis, IN) to PCR reactions, the enzyme was preincubated with the TaqStart antibody (ProMega, Madison, WI) according to manufacturer's instructions. Radioactive PCR reactions were analyzed on 5% nondenaturing polyacrylamide gels, dried, and exposed to Molecular Dynamics phosphorimager screens (Sunnyvale, CA) for 1 h. Screens were scanned with a Molecular Dynamics Storm 860, and band intensities were quantified using ImageQuant software. The gene expression levels were represented as intensity ratio of OCT-4 or hTERT to 18S.

TRAP assay. Telomerase activity was assayed by telomeric repeat amplification protocol (TRAP)[14,15].

Differentiation *in vitro*. Cells were dissociated into small clumps by collagenase IV, cultured in suspension as embryoid bodies in differentiation medium for four days, transferred onto gelatin-coated plates, and cultured for an additional seven days. Differentiation medium contains 80% KO-DMEM, 20% FBS (not heat-inactivated), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids.

Teratoma formation. Cells were injected intramuscularly into SCID/beige mice ($\sim 5 \times 10^6$ cells per site). After 78–84 days, tumors were processed for histological analysis performed by IDEXX Corp. (West Sacramento, CA).

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A method for the amidation of recombinant peptides expressed as intein fusion proteins in *Escherichia coli*

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The increasing use of peptides as pharmaceutical agents, especially in the antiviral and anti-infective therapeutic areas, requires cost-effective production on a large scale[1]. Many peptides need carboxy amidation for full activity or prolonged bioavailability[2]. However, this modification is not possible in prokaryotes and must be done using recombinant enzymes[3] or by expression in transgenic milk[4]. Methods employing recombinant enzymes are appropriate for small-scale production, whereas transgenic milk expression is suitable for making complex disulfide-containing peptides required in large quantity. Here we describe a method for making amidated peptides using a modified self-cleaving vacuolar membrane ATPase (VMA) intein expression system[5]. This system is suitable for making amidated peptides at a laboratory scale using readily available constructs and reagents. Further improvements are possible, such as reducing the size of the intein to improve the peptide yields (the VMA intein comprises 454 amino acids) and, if necessary, secreting the fusion protein to ensure correct N-terminal processing to the peptide. With such developments, this method could form the basis of a large-scale cost-effective system for the bulk production of amidated peptides without the use of recombinant enzymes or the need to cleave fusion proteins.

Inteins are a family of proteins that can seamlessly excise themselves from between two protein "exteins", which are subsequently joined to give a single polypeptide (Fig. 1A). The key to the intein splicing

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